Cell, Vol. 107, 309–321, November 2, 2001, Copyright ©2001 by Cell Press

ATP Requirements and Small Interfering RNA Structure in the RNA Interference Pathway

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Summary

We examined the role of ATP in the RNA interference (RNAi) pathway. Our data reveal two ATP-dependent steps and suggest that the RNAi reaction comprises at least four sequential steps: ATP-dependent processing of double-stranded RNA into small interfering RNAs (siRNAs), incorporation of siRNAs into an inactive ~360 kDa protein/RNA complex, ATP-dependent unwinding of the siRNA duplex to generate an active complex, and ATP-independent recognition and cleavage of the RNA target. Furthermore, ATP is used to maintain 5′ phosphates on siRNAs. A 5′ phosphate on the target-complementary strand of the siRNA duplex is required for siRNA function, suggesting that cells check the authenticity of siRNAs and license only bona fide siRNAs to direct target RNA destruction.

Introduction

In animals, double-stranded RNA (dsRNA) specifically silences expression of a corresponding gene, a phenomenon termed RNA interference (RNAi; Fire et al., 1998; Montgomery et al., 1998). One function of the RNAi machinery is to maintain the integrity of the genome by suppressing the mobilization of transposons and the accumulation of repetitive DNA (Jensen et al., 1999; Ketting et al., 1999; Ketting and Plasterk, 2000; Malinsky et al., 2000). The RNAi machinery may also defend cells against viral infection (reviewed in Montgomery and Fire, 1998; Fire, 1999; Hunter, 1999; Li and Ding, 2001; Sharp, 2001) and regulate expression of cellular genes (Smardon et al., 2000; Aravin et al., 2001).

RNAi bears a striking similarity to posttranscriptional cosuppression in plants (posttranscriptional gene silencing, PTGS; Baulcombe, 1999; Grant, 1999; Ratcliff et al., 1999) and *Neurospora crassa* (quelling; Cogoni et al., 1994, 1996). Genetic screens in worms, fungi, plants, and green algae have identified genes required for RNAi or PTGS (Cogoni and Macino, 1997, 1999; Elmayan et al., 1998; Tabara et al., 1999; Fagard et al., 2000; Wu-Scharf et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000), and the RNAi and cosuppression pathways in *Caenorhabditis elegans*, *Neurospora*, and *Arabidopsis thaliana* require some of the same genes (Catalanotto et al., 2000; Dernburg et al., 2000; Fagard et al., 2000; Ketting and Plasterk, 2000; Hammond et al., 2001b). Mutations in a subset of these genes permit the mobilization of transposons (Ketting et al., 1999; Tabara et al., 1999; Grishok et al., 2000), whereas a second class of mutants, including the *rde-1* and *rde-4* loci, are defective for RNAi but show no other phenotypic abnormalities (Tabara et al., 1999). Rde-1 is a member of the PPD family of proteins (*P*AZ and *P*IWI *D*omain), which are characterized by an N-terminal "Piwi/Argonaute/Zwille" (PAZ) domain and a C-terminal 'PIWI' domain (Cerutti et al., 2000). PPD proteins are required not only for RNAi in worms (Rde-1; Tabara et al., 1999) and flies (Ago-2; Hammond et al., 2001a), but also for PTGS in plants (AGO-1; Fagard et al., 2000) and quelling in fungi (Qde-1; Catalanotto et al., 2000).

In vitro, dsRNA targets mRNA for cleavage in lysates of early *Drosophila* embryos or extracts of cultured *Drosophila* S2 cells (Tuschl et al., 1999; Hammond et al., 2000; Zamore et al., 2000). RNAi in vitro requires ATP (Zamore et al., 2000). The molecular basis for the ATP requirement is due, in part, to a requirement for ATP in the initial processing of long dsRNA into the 21–25 nt small interfering RNAs (siRNAs) which guide target cleavage (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Elbashir et al., 2001a; Bernstein et al., 2001). siRNAs have been detected in vivo in plants (Hamilton and Baulcombe, 1999; Hutvágner et al., 2000), flies (Yang et al., 2000), worms (Parrish et al., 2000), and trypanosomes (Djikeng et al., 2001). Recent studies with synthetic RNA duplexes demonstrate that each siRNA duplex cleaves the target RNA at a single site (Elbashir et al., 2001a). 2- or 3-nt overhanging 3′ ends within the siRNA duplex are required for efficient target cleavage (Elbashir et al., 2001a). Such 3′ overhangs are characteristic of the products of an RNase III cleavage reaction, and, in cultured *Drosophila* S2 cells, cleavage of the dsRNA into siRNAs requires the multidomain RNase III enzyme, Dicer (Bernstein et al., 2001). Intriguingly, Dicer interacts directly or indirectly with Ago-2, a PPD protein required for RNAi in cultured *Drosophila* S2 cells (Hammond et al., 2001a). Dicer orthologs are found in the genomes of plants (Jacobsen et al., 1999), worms, fission yeast, and humans (Matsuda et al., 2000; Bernstein et al., 2001). In worms, the Dicer ortholog, Dcr-1, is required both for RNAi (Knight and Bass, 2001) and for the maturation of small temporal RNAs (stRNAs), single-stranded 21–22 nt RNAs that control the timing of development (Grishok et al., 2001). Dicer in flies and humans is likewise responsible for generating the *let-7* stRNA (Hutvágner et al., 2001).

Here, we dissect the role of ATP in the RNAi pathway. Our data suggest that the RNAi reaction comprises at least four sequential steps: ATP-dependent dsRNA processing, ATP-independent incorporation of siRNAs into an inactive ~360 kDa complex, ATP-dependent unwinding of the siRNA duplex, and ATP-independent recognition and cleavage of the target RNA. Remarkably, a 5′ phosphate is required for entry of an siRNA into the RNAi pathway. In vitro, this phosphate is maintained by a kinase which can recognize authentic siRNAs. Furthermore, the phosphorylation status of the 5′ ends of an siRNA duplex is monitored by the RNAi machinery, sug-

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gesting that cells check the authenticity of siRNAs, ensuring that only bona fide siRNAs direct target RNA destruction.

Results and Discussion

ATP and dsRNA Processing

In vitro and in vivo studies suggest that RNAi is a multistep process that begins with the ATP-dependent processing of long dsRNA into 21–23 nt siRNAs by Dicer protein, perhaps in conjunction with other proteins. In *Drosophila* embryo lysates, siRNAs are produced to 6-fold higher levels in high ATP concentrations than in low (Zamore et al., 2000), and dsRNA cleavage by immunoprecipitated Dicer protein requires ATP (Bernstein et al., 2001). As a starting point for defining the role of ATP in the RNAi pathway, we reexamined the ATP dependence of siRNA production during the initial phase of the cleavage reaction, when the rate of dsRNA cleavage is linear (Figure 1A). A prior study assessed siRNA production at steady state (Zamore et al., 2000). The experiments presented here employed a revised ATP depletion strategy that reduced ATP-levels by at least 5,000-fold, to <100 nM. During the first 16 min of incubation of a uniformly ^{32}P-radiolabeled dsRNA derived from the *Renilla reniformis* luciferase (*Rr*-luc) gene, the rate of siRNA production was ~47-fold faster in the presence of ATP and an ATP-regenerating system (filled squares) than in their absence (open squares). In the presence of ATP, siRNA production increased dramatically after about 4 min incubation. This initial lag might reflect the ATP-dependent rearrangement of the dsRNA and/or the proteins required to produce siRNAs, such as Dicer. Consistent with this idea, we did not detect such an initial lag phase for siRNA production in the absence of ATP. siRNA production in the presence of ATP required incubation at 25°C; no siRNAs were produced in the presence of ATP at 4°C (filled circles). These data quantitatively confirm previous observations that cleavage of the dsRNA into siRNAs requires ATP (Zamore et al., 2000; Bernstein et al., 2001).

siRNA Duplexes Are Bona Fide Intermediates in the RNAi Pathway

RNAi can be initiated with synthetic siRNAs in vitro and in vivo, suggesting that siRNAs are intermediates in the RNAi reaction (Elbashir et al., 2001a, 2001b; Caplen et al., 2001). However, when purified from a denaturing gel, siRNAs generated by cleavage of dsRNA reduced target mRNA expression by only ~2-fold (unpublished data). To assess directly if the products of dsRNA cleavage—native siRNAs—are true intermediates in the RNAi reaction, we developed an isolation procedure designed to preserve their proposed double-stranded character. Uniformly ^{32}P-radiolabeled *Rr*-luc dsRNA was incubated in a standard RNAi reaction in the absence of target mRNA, deproteinized, and fractionated by gel filtration. Two peaks of radioactivity eluted from the column (Figure 1C, black circles). The first corresponded to unprocessed dsRNA; the second peak contained native siRNAs. The elution position of the native siRNAs from the gel-filtration column coincided with that of a synthetic siRNA duplex (red triangles), but not a single-stranded 21 nt RNA (blue squares). Thus, the siRNAs produced by Dicer-mediated cleavage of long dsRNA are double-stranded.

The gel filtration procedure was repeated with unlabeled *Rr*-luc dsRNA processed in vitro into native siRNAs, and the column fractions assessed for their ability to mediate sequence-specific interference when added to lysate in the presence of ATP. Two peaks of interfering activity were detected (Figure 1D). The first peak, fractions 3–13, corresponded to unprocessed dsRNA and served as an internal control for the experiment. The second peak, fractions 41–50, corresponded to the native siRNAs. None of the column fractions exhibited any significant degradation of an unrelated *Photinus pyralis* luciferase (*Pp*-luc) mRNA (Figure 1E). Preheating the purified native siRNAs to 95°C for 5 min abolished their ability to initiate interference, suggesting that only double-stranded siRNAs can enter the pathway (data not shown). These results, together with those of Tuschl and coworkers (Elbashir et al., 2001a), demonstrate that siRNAs are true intermediates in RNAi, not the products of an off-pathway side reaction. Furthermore, they support the original proposal of Hamilton and Baulcombe that siRNAs are the specificity determinate for both PTGS and RNAi (Hamilton and Baulcombe, 1999).

A Second ATP-Dependent Step in RNAi

In performing the experiments shown in Figures 1D and 1E, we observed that none of the column fractions mediated sequence-specific interference when incubated with lysate in the absence of ATP. This observation was expected for the first peak (fractions 3–13), since dsRNA cleavage into siRNAs requires ATP, but unexpected for the second peak, which corresponds to fully processed, native siRNAs. Therefore, we asked if interference mediated by siRNAs, rather than dsRNA, also required ATP. Native siRNAs isolated by gel-filtration were added to a standard RNAi reaction in the presence or absence of ATP (Figure 2) and incubated at room temperature for 30 min, and then a *Rr*-luc target mRNA was added. In the presence of both native siRNAs and ATP, the target RNA was rapidly degraded (native, +ATP). In contrast, only nonspecific degradation of the target RNA occurred in the absence of ATP (native, −ATP). These results point to the existence of a second ATP-dependent step, downstream in the RNAi reaction from dsRNA processing. However, an alternative explanation is that undetected dsRNA contaminated the native siRNA preparation. Since dsRNA processing requires ATP, this could, in principle, explain the apparent ATP requirement for native siRNA-directed interference. To exclude this possibility, we assessed the ATP dependence of interference directed by a chemically synthesized siRNA duplex targeting the *Pp*-luc mRNA (Figure 2). In the absence of ATP, no sequence-specific interference occurred (synthetic, −ATP). Addition of ATP and an energy regenerating system only partially restored normal ATP levels, because of the high concentrations of glucose used to deplete ATP. Under these conditions, partial interference was observed (synthetic, ATP rescue). In contrast, in a standard RNAi reaction containing 1 mM ATP, synthetic siRNAs mediated potent interference (synthetic, +ATP). Therefore, interference by both native

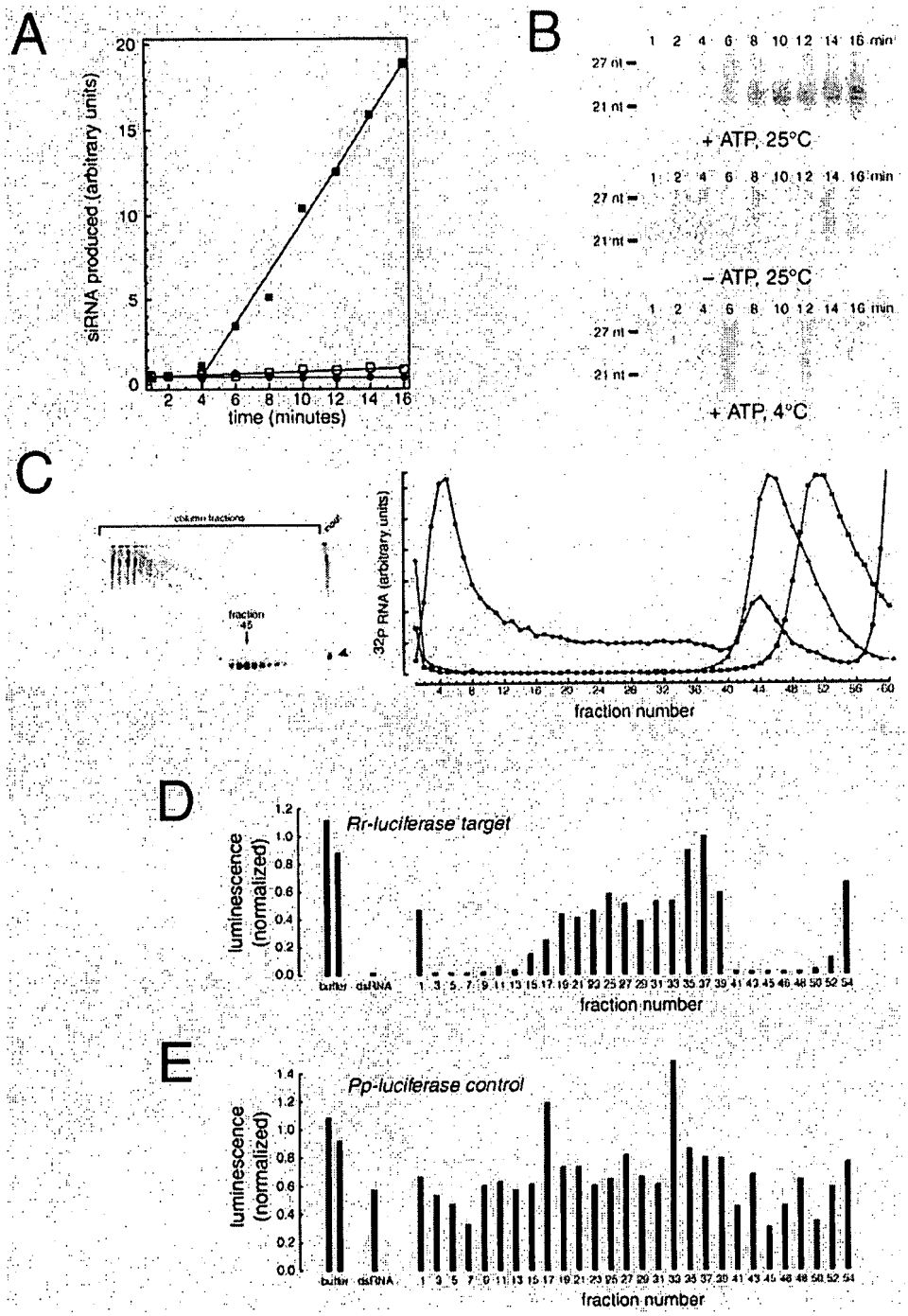

Figure 1. Production of the siRNAs that Mediate Sequence-Specific Interference Requires ATP

(A) Measurement of the initial rate of siRNA production from uniformly ^{32}P-radiolabeled 501 bp *Rr*-luc dsRNA in the presence of 1 mM ATP at 25°C (filled squares) or 4°C (filled circles), or at 25°C in the absence of ATP (open squares).

(B) The data presented graphically in (A).

(C) Isolation of native siRNAs by gel filtration. Uniformly ^{32}P-radiolabeled 501 bp *Rr*-luc dsRNA was processed in a standard RNAi reaction, deproteinized, and fractionated on a Superdex-200 gel filtration column. Fractions were analyzed by electrophoresis on a 15% acrylamide sequencing gel (left panel) and by scintillation counting (right panel, black circles). Double-stranded (red triangles) and single-stranded (blue squares) synthetic siRNAs were chromatographed as standards. The native siRNA peak and the synthetic siRNA duplex marker do not precisely comigrate, likely because the native siRNAs are a mixture of 21- and 22-nt species (Zamore et al., 2000; Elbashir et al., 2001a) and the synthetic siRNAs are 21 nt.

(D and E) Analysis of each column fraction for RNAi activity in an in vitro reaction containing both *Rr*-luc and *Pp*-luc mRNAs. Luminescence was normalized to the average of the two buffer controls.

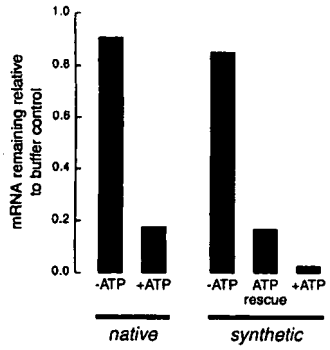

Figure 2. RNAi Mediated by siRNAs Requires ATP

Native siRNAs targeting *Rr*-luc were assayed for RNAi against an *Rr*-luc RNA target. A synthetic siRNA duplex targeting *Pp*-luc was tested for RNAi against a *Pp*-luc mRNA.

and synthetic siRNAs requires ATP, revealing one or more novel ATP-dependent step(s) distinct from the ATP-dependent cleavage of long dsRNA into siRNAs.

Target Recognition and Cleavage Are ATP-Independent

One possible source for the ATP requirement might be that target cleavage, like the cleavage of dsRNA into siRNAs by Dicer, requires ATP. To test if either target recognition or cleavage requires ATP, we incubated a 501 bp *Rr*-luc or a 505 bp *Pp*-luc dsRNA in a standard RNAi reaction to permit its processing into native siRNAs, then removed ATP from the reaction and evaluated its ability to cleave a corresponding target RNA (Figure 3). Two different strategies were employed to remove ATP from the reaction after the initial dsRNA processing step. In both strategies, the interfering RNA—dsRNA or siRNA—was preincubated with lysate in the presence of ATP, then ATP was removed from the reaction, and finally, the target RNA was added in the presence or absence of ATP. In the first strategy (Figure 3A), the ATP regenerating enzyme, creatine kinase, was inactivated with *N*-ethylmaleimide (NEM; Worthington, 1988), unreacted NEM quenched, and ATP depleted with hexokinase and glucose (Figure 3B, filled symbols). Then, a *Pp*-luc target mRNA was added to the reaction. Lysate treated with NEM, then DTT, prior to the addition of the creatine kinase supported RNAi (data not shown). In a separate series of controls, DTT was added prior to the NEM, and no hexokinase was added (open symbols). High ATP levels were maintained during the dsRNA processing portion of the experiment and throughout the experiment when DTT was added prior to NEM. However, when the reactions were sequentially treated with NEM, DTT, then hexokinase plus glucose, ATP levels were reduced ~5,000-fold to ≤100 nM. In all conditions in which the 505 bp *Pp*-luc dsRNA was included (triangles), the *Pp*-luc target mRNA was cleaved regardless of the ATP concentration at the time of target RNA addition. Under all conditions, interference remained sequence-specific: a 501 bp *Rr*-luc dsRNA did not affect the stability of the *Pp*-luc mRNA target in the presence (open circles) or absence of ATP (filled circles). Furthermore, when a synthetic siRNA duplex targeting the *Pp*-luc mRNA was first preincubated with lysate and ATP, the *Pp*-luc target mRNA was subsequently cleaved both in the presence (open squares) and absence (filled squares) of ATP. These results strongly argue that neither target recognition nor target cleavage requires ATP as a cofactor, and they suggest that ATP participates in a step prior to the encounter of the siRNA with its RNA target.

Nonetheless, the experiments shown in Figure 3B cannot exclude a requirement for some other small molecule cofactor (e.g., GTP) in target recognition and cleavage. To examine this possibility, we employed a second strategy to remove ATP and other small molecule cofactors (Figure 3C). A 501 bp *Rr*-luc dsRNA was incubated with lysate and ATP in a standard RNAi reaction, then the siRNA/protein complex was precipitated with ammonium sulfate. The resolubilized ammonium sulfate precipitate was extensively dialyzed to remove small molecule cofactors, then treated with hexokinase and glucose to further deplete ATP. The procedure reduced the initial 1 mM ATP concentration to ≤50 nM (data not shown). Furthermore, the dialysis step is expected to have significantly reduced endogenous pools of other nucleotide tri-, di-, and monophosphate cofactors. Finally, the ATP-depleted siRNA/protein complex was tested for cleavage of a 501 nt *Rr*-luc target RNA or an unrelated 441 nt control RNA in the presence or absence of ATP (Figure 3D). The *Rr*-luc dsRNA directed efficient target recognition and cleavage in the absence of exogenous ATP. In both the presence and absence of ATP, target cleavage was specific for the *Rr*-luc target mRNA; the control RNA was not cleaved. For ATP or some other small molecule cofactor to be involved in these steps in the RNAi reaction, it would have had to remain associated with the RNAi machinery through 16 hr of dialysis against multiple changes of a 5,000-fold excess of buffer. The simplest explanation is that both target recognition and target cleavage are ATP-independent steps.

The ATP-dependent step identified by the experiments in Figure 2 therefore lies downstream of dsRNA processing but upstream of target recognition. We can envision several types of ATP-dependent steps that might explain our findings. Formation of an siRNA/protein complex might require ATP. Alternatively, association of proteins with the siRNAs might be ATP-independent, but a conformational change in the siRNA itself might require ATP. For example, an ATP-dependent RNA helicase might unwind the two strands of the siRNA prior to its encounter with the target RNA. In support of this idea, proteins with the signature motifs of ATP-dependent RNA helicases have been implicated in RNAi in flies and worms (Bernstein et al., 2001; Knight and Bass, 2001), PTGS in *Chlamydomonas reinhardtii* and plants (Wu-Scharf et al., 2000; Dalmay et al., 2001), and *Stellate* silencing in flies (Aravin et al., 2001).

siRNA-Protein Complex Formation

To detect the formation of a protein complex on siRNAs, uniformly ^{32}P-radiolabeled 501 nt *Rr*-luc dsRNA was incubated in a standard RNAi reaction in the absence of target to permit its cleavage into siRNAs and to permit the resulting siRNAs to assemble into a protein/siRNA

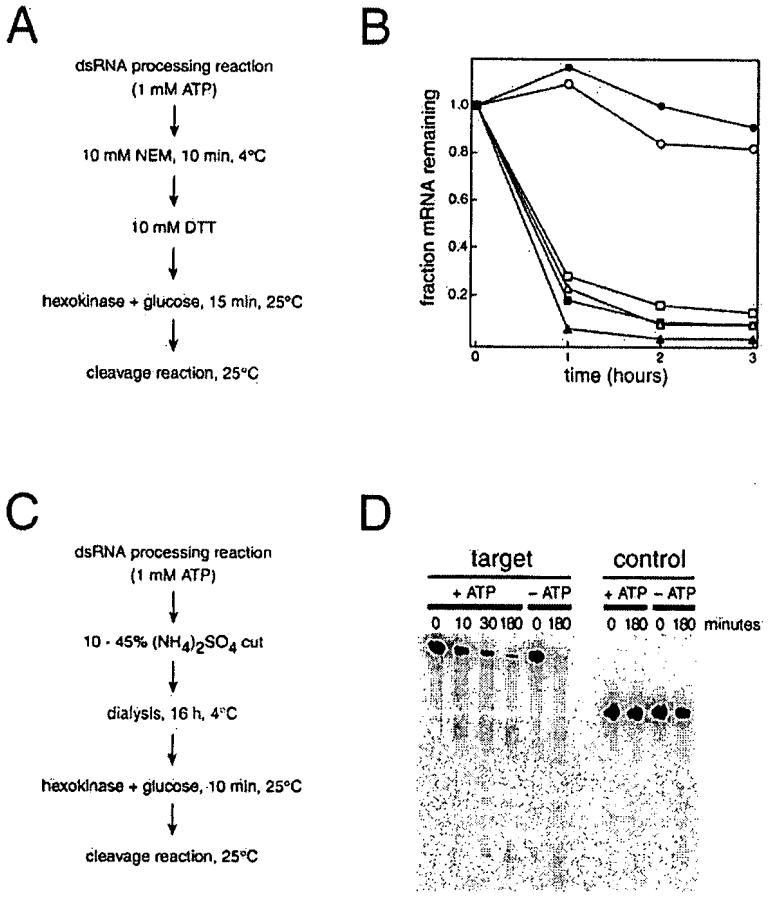

Figure 3. Target Recognition and Cleavage Is ATP-Independent

(A) Scheme for depleting ATP from the RNAi reaction after an initial preincubation in the presence of ATP.

(B) After 30 min preincubation with ATP of a 505 bp *Pp*-luc dsRNA (triangles) or a 501 bp *Rr*-luc dsRNA (circles), ATP was depleted by the sequential addition of NEM, DTT, and hexokinase plus glucose (filled symbols), then a *Pp*-luc target mRNA added. In the controls, DTT was added before NEM, and hexokinase was omitted (open symbols). A synthetic siRNA duplex targeting the *Pp*-luc mRNA was also tested (squares). In all cases, the target RNA was a *Pp*-luc mRNA.

(C) Alternative scheme for depleting ATP from the RNAi reaction.

(D) After preincubation of a *Rr*-luc dsRNA with lysate and ATP, recognition and cleavage of a *Rr*-luc target RNA or a 441 nt control RNA was measured in the presence or the absence of ATP.

complex (siRNP). After 2 hr incubation, the reaction was chromatographed on a Superdex-200 gel filtration column. siRNAs were predominantly associated with a ~360 kDa siRNP (Figure 4A). Formation of the siRNP complex required protein, since it was not observed when the complex was treated with proteinase K prior to gel filtration (Figure 1C). A second peak of ^{32}P-radiolabled siRNAs coincides with siRNAs unbound by protein (compare Figures 1C and 4A), indicating that a significant fraction of the siRNAs generated by dsRNA processing in vitro do not stably associate with protein. Next, purified, ^{32}P-native siRNAs were incubated with lysate either in the presence (Figure 4B) or the absence (Figure 4C) of ATP. The same two siRNA-containing peaks were observed: a ~360 kDa siRNP (peaking in fractions 18–20) and native siRNAs not associated with protein (peaking in fractions 42–46). Thus, assembly of the siRNP does not require ATP.

Recently, Hannon and coworkers reported isolation of an siRNA-containing, ~500 kDa complex from cultured *Drosophila* S2 cells (Hammond et al., 2001a). This ~500 kDa complex contained the target-cleaving nuclease (Hammond et al., 2001a). To assess the capacity of the ~360 kDa siRNP to direct target cleavage, we incubated a single synthetic siRNA duplex in the *Drosophila* embryo lysate in the presence of ATP, then fractionated the reaction by gel filtration on Superdex-200. The siRNA duplex was chosen because it directs cleavage of the 510 nt *Pp*-luc target RNA at a single site, 72 nt from the 5′ cap, yielding a 72 nt RNA product diagnostic of RNAi activity (Elbashir et al., 2001a). In these experiments, the synthetic siRNA duplex was 3′ radiolabeled on the sense siRNA strand. Control experiments showed that such a 3′ modified siRNA duplex does not impair the ability of the siRNA to mediate RNAi (data not shown). Thus, the 510 nt target RNA, the 72 nt RNAi cleavage product, and the siRNA itself can be detected simultaneously on a denaturing acrylamide gel. The ability of each gel filtration column fraction to support sequence-specific target cleavage was assessed (Figure 4D). A major peak of radiolabeled siRNA was detected in fractions 18–22, indicating formation of the siRNP complex. Although this siRNP complex contains virtually all of the siRNAs associated with protein, it was not competent to cleave a target RNA. Instead, two peaks of RNAi activity were observed: one in the void volume of the column (fractions 2–6), and a broad peak of RNAi activity of apparent molecular weight <232 kDa (fractions 26–40). Surprisingly, these peaks contained barely detectable levels of siRNA, but were nonetheless nearly as active as the unfractionated reaction, despite having suffered dilution from the gel filtration chromatography (Figure 4D, compare the amount of ^{32}P-siRNA in the input to the column [lane labeled "siRNA"] to that in the active column fractions). Control experiments (not shown) demonstrated that little if any degradation of siRNA duplexes occurs in the lysate, indicating that the distribution of ^{32}P-siRNAs accurately reflects the distri-

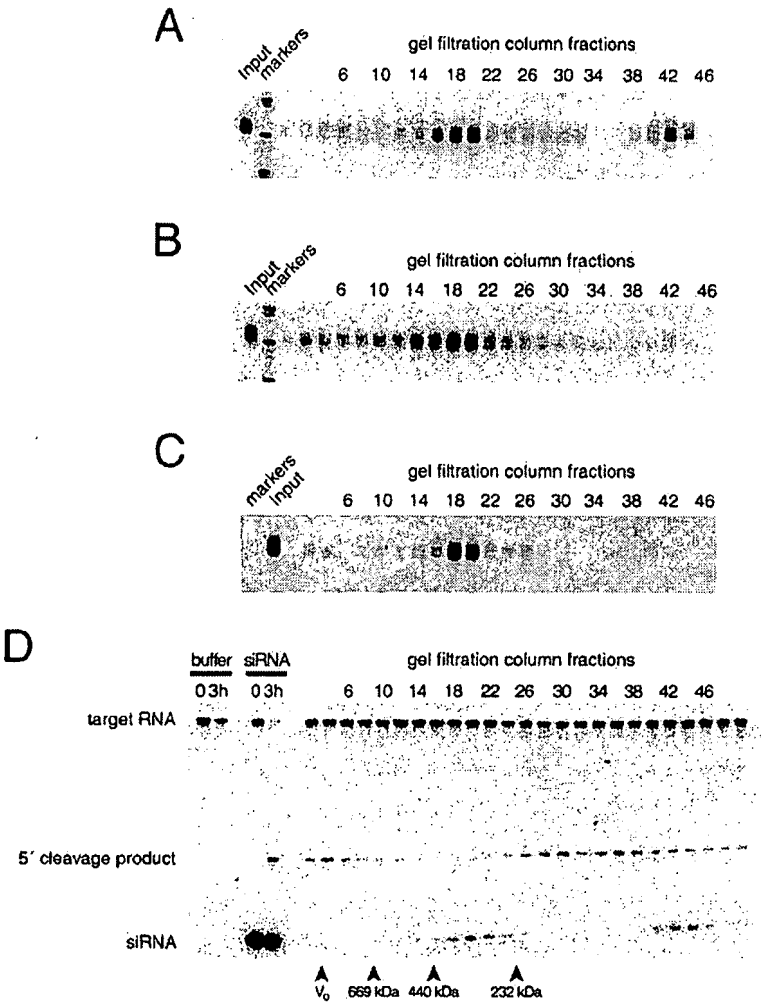

Figure 4. siRNP Complex Formation and Activity

(A) Analysis of siRNP formation during the processing of a 501 *Rr*-luc dsRNA into native siRNAs in the presence of ATP.

(B) Analysis of siRNP formation in the presence of ATP for purified, native siRNA duplexes.

(C) As in (B), except in the absence of ATP.

(D) RNAi activity of gel filtration fractions prepared as in (B), but using a synthetic siRNA duplex targeting the *Pp*-luc mRNA. The siRNA was incubated in an RNAi reaction for 1 hr, then fractionated by gel filtration. A sample of the input to the column was used in a control reaction (siRNA); every other column fraction was analyzed for RNAi activity. The elution position of molecular weight markers for all four panels is shown in (D). V_o, void volume; 669 kDa, Thyroglobulin; 440 kDa, Ferritin; 232 kDa, Catalase.

bution of siRNA in the column fractions. Thus, the majority of siRNAs associated with protein are present in a ~360 kDa siRNP complex that is not competent to cleave a target RNA, whereas a minority of the siRNAs are in a smaller, highly active complex. Mixing experiments (not shown) demonstrated that the fraction containing the ~360 kDa siRNP does not contain an inhibitor of RNAi. We defer to below the question of whether the ~360 kDa complex is a productive intermediate in the RNAi pathway or a nonproductive, off-pathway intermediate.

siRNA Unwinding during the RNAi Reaction

To test if ATP is used during the RNAi reaction to separate the two strands of the siRNA duplex, we developed a method to differentiate siRNA duplexes from single-stranded siRNAs (Figure 5A). In this assay, RNAi was initiated in vitro with a synthetic siRNA duplex in which the sense strand was 3′ radiolabeled. The RNAi reaction was quenched by the simultaneous addition of an SDS-containing stop buffer, proteinase K, and a 25-fold molar excess of an unlabeled competitor RNA containing the sequence of the 19 paired nucleotides from the sense strand of the siRNA duplex. The samples were analyzed by nondenaturing acrylamide gel electrophoresis.

We used the assay to assess if the siRNA duplex is a substrate for ATP-dependent helicase proteins in the *Drosophila* embryo lysate. Unwinding of the siRNA duplex was monitored in an RNAi reaction in the presence and absence of ATP. No siRNA unwinding was detected in the absence of ATP, whereas a small percent (≤5%) of unwound siRNA was detected with ATP (Figure 5B). Furthermore, the siRNA was almost entirely double-stranded in the inactive ~360 kDa siRNP complex (Figure 5C). In contrast, single-stranded siRNA resided in the same two peaks that showed RNAi activity in the target cleavage assay: the void volume of the column and a peak of apparent molecular weight <232 kDa. These data suggest (1) that RNAi activity is associated with a population of siRNAs that are unwound and (2) that a protein-siRNA complex of <232 kDa contains all of the factors required for efficient, sequence-specific target cleavage. We propose that this complex represents the minimal, active RNA-induced silencing complex (RISC; Hammond et al., 2000). We term this complex the RISC*. These results are at odds with previous findings that the active RISC is a ~500 kDa complex (Hammond et al., 2001a). A possible explanation is that our chromatographic procedure resolved the smaller active complex from a larger precursor complex, but that these

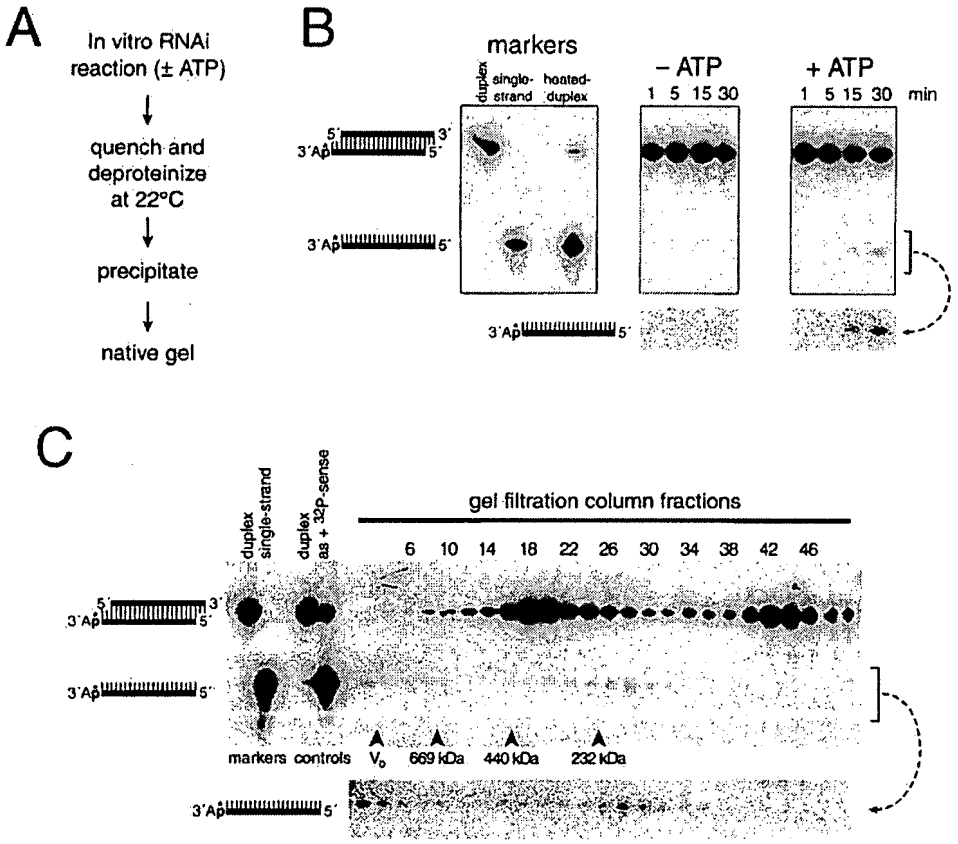

Figure 5. ATP-Dependent siRNA Unwinding Correlates with RNAi Activity

(A) Outline of the assay used in (B) and (C).

(B) Native acrylamide gel analysis of siRNA unwinding in the absence and presence of ATP. An overexposure of the region of the gel corresponding to single-stranded siRNA is shown in the lower panel.

(C) Analysis of siRNA unwinding for the gel filtration fractions from Figure 4D. An overexposure of the region of the gel corresponding to single-stranded siRNA is shown in the lower panel. Molecular weight standards are as in Figure 4D.

two species remain associated in a single, ~500 kDa complex under other conditions. Alternatively, a dissociable cofactor, such as an ATP-dependent RNA helicase, might be present in fractions 26–40. This factor might support RNAi by acting on a small amount of the inactive ~360 kDa complex present in these fractions, converting it to the RISC* by unwinding the siRNA duplex.

5′ Phosphorylation Status and siRNA Activity

Synthetic siRNAs bearing 5′ hydroxyl termini have been used successfully to initiate interference in *Drosophila* embryo lysates (Elbashir et al., 2001a) and in cultured mammalian cells (Elbashir et al., 2001b). Nonetheless, native siRNAs, generated by cleavage of dsRNA, contain 5′ phosphate ends (Elbashir et al., 2001a). Therefore, we asked if a 5′ phosphate is merely a consequence of the enzymatic mechanism of dsRNA cleavage by the RNase III enzyme Dicer, or if it is an essential feature of an active siRNA. We first examined the 5′ phosphorylation status of the anti-sense strand of a synthetic siRNA duplex that contained 5′ hydroxyl groups on both strands. Figure 6A shows that upon incubation in the lysate with ATP, the siRNA was rapidly phosphorylated, so that after 15 min nearly all of the synthetic siRNA had a 5′ phosphate group (lysate, HO-rU). This finding was surprising, because *Drosophila* embryo lysates contain a potent phosphatase activity that rapidly dephosphorylated exogenous 5′ ^{32}P-radiolabeled siRNA duplexes (data not shown). The 5′ phosphate of the siRNA must be in rapid exchange, but the sum of the rates of phosphatase and kinase activities produces an siRNA bearing a 5′ phosphate at steady-state. Therefore, both synthetic (5′ hydroxyl) and native (5′ phosphate) siRNAs are expected to exist predominantly as 5′ phosphorylated species in the in vitro RNAi reaction, and perhaps in vivo as well.

To assess if a 5′ phosphate is required for RNAi, we designed an siRNA duplex in which the 5′ end of the anti-sense strand was blocked by replacing the 5′ hydroxyl with a 5′ methoxy group (CH_3O). In order to facilitate chemical synthesis of the 5′ block, the first nucleotide of the anti-sense siRNA strand, uracil, was replaced with 2′ deoxythymidine (dT). An siRNA duplex in which the 5′ terminus was a hydroxyl group, but the first nucleotide was dT, was prepared in parallel. The 5′ blocked (CH_3O-dT) and 5′ dT (HO-dT) anti-sense siRNA strands were each annealed to a standard, 5′ hydroxyl sense

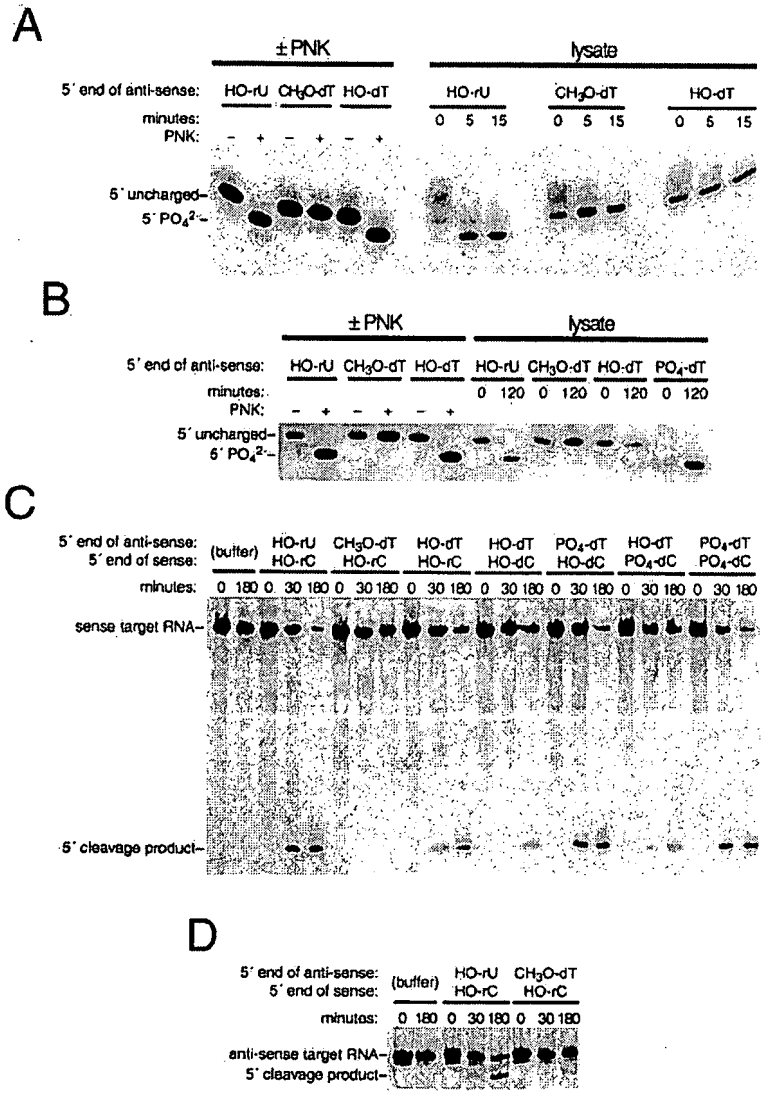

Figure 6. 5′ Phosphates Are Critical Determinants of siRNA Activity

(A and B) Phosphorylation status of the anti-sense strand of synthetic siRNA duplexes upon incubation with polynucleotide kinase (PNK) or *Drosophila* embryo lysate.

(C) RNAi activity of synthetic siRNA duplexes measured for a sense *Pp*-luc RNA target.

(D) RNAi activity of synthetic siRNA duplexes measured for an anti-sense *Pp*-luc RNA target.

strand (HO-rC). The 5′ blocked siRNA was not phosphorylated after incubation with either polynucleotide kinase (PNK) or lysate (CH_3O-dT; Figures 6A and 6B). Surprisingly, the siRNA bearing a 5′ dT on the anti-sense strand (HO-dT) was a poor substrate for phosphorylation in the lysate, despite being a good substrate for PNK (Figure 6A): it was not detectably phosphorylated after 15 min in the lysate, although a small fraction was phosphorylated after 2 hr (HO-dT; Figures 6A and 6B). These data show that *Drosophila* embryos contain a nucleic acid kinase that discriminates against 5′ deoxy siRNAs.

Next, we examined the capacity of these siRNA duplexes to trigger RNAi. This siRNA sequence directs cleavage of the 510 nt sense *Pp*-luc target RNA to yield a diagnostic 72-nt 5′ product (Elbashir et al., 2001a and Figure 5D). As expected, the standard siRNA (anti-sense, HO-rU; sense, HO-rC) directed efficient cleavage of the target RNA (Figure 6C), as evidenced by the disappearance of the 510 nt RNA and the appearance of the 72 nt RNA. In contrast, no target cleavage was detected for the 5′ blocked anti-sense siRNA (CH_3O-dT) paired with a standard sense siRNA strand (HO-rC). These data suggest that a 5′ phosphate is required on the siRNA strand that guides target cleavage. This hypothesis predicts that anti-sense siRNAs that are poorly phosphorylated in the lysate will be poor effectors of sense target cleavage. Consistent with the prediction, an siRNA in which the anti-sense strand is 5′ hydroxyl, 5′ dT (HO-dT), which is inefficiently phosphorylated in the lysate (Figures 6A and 6B), is less efficient in directing sense target cleavage than a standard siRNA (Figure 6C). To test if the defect was a direct consequence of the inefficiency with which the 5′ dT anti-sense RNA was phosphorylated, a 5′ dT anti-sense RNA bearing a 5′ phosphate was annealed to a 5′ hydroxyl, 5′ dC sense siRNA. Like the 5′ dT modification on the anti-sense strand, 5′ dC on the sense strand inhibits phosphorylation by the kinase in the lysate (data not shown). Use of a 5′ dC sense strand, therefore, allowed us to examine the effect of a 5′ phosphate on the 5′ dT, anti-sense strand in an siRNA duplex in which the sense strand was predominantly 5′ hydroxyl. This siRNA duplex (anti-sense, PO_4-dT; sense, HO-dC) was as efficient in cleaving the sense target RNA as a standard, siRNA duplex (anti-sense,

HO-rU; sense, HO-rC; Figure 6C). Thus, the sole defect caused by a 5′ dT anti-sense strand is that it is a poor kinase substrate in the lysate. An siRNA in which both strands were 5′ phosphorylated and 5′ deoxy (anti-sense, PO_4-dT; sense, PO_4-dC) was no more efficient than the siRNA comprising a 5′ phosphate, 5′ dT anti-sense strand and a 5′ hydroxyl, 5′ dC sense strand (anti-sense, PO_4-dT; sense, HO-dC; Figure 6C). As expected, an siRNA in which both strands were 5′ deoxy and 5′ hydroxyl (anti-sense, HO-dT; sense, HO-dC) was defective in sense target cleavage. This defect was not remedied by adding a phosphate to the sense strand (anti-sense, HO-dT; sense, PO_4-dC), lending further support to the idea that a 5′ phosphate on the anti-sense strand is required to guide sense target cleavage (Figure 6C). We conclude that a 5′ phosphate on the guide strand of an siRNA is required for RNAi. We note that RNAi directed by an siRNA in which both strands are 5′ phosphate but also 5′ deoxy), nonetheless required ATP (data not shown). This ATP-requirement likely reflects the role of ATP in siRNA unwinding (see above) rather than in 5′ phosphorylation.

In the course of these experiments, we observed that an siRNA in which both strands were 5′ hydroxyl and 5′ deoxy was slightly worse at guiding sense target cleavage than the 5′ hydroxyl siRNA in which only the anti-sense strand was 5′ deoxy. Therefore, we asked directly if the 5′ phosphate of the nonguiding strand is also important for siRNA function. We examined cleavage of an anti-sense target RNA for the siRNA with a 5′ methoxy anti-sense strand and a standard sense strand (anti-sense, CH_3O-dT; sense, HO-rC). With respect to an anti-sense target RNA, this siRNA duplex is blocked for 5′ phosphorylation only on the nonguiding strand. As expected, a standard siRNA (anti-sense, HO-rU; sense, HO-rC) cleaved a 510 nt, anti-sense *Pp*-luc target RNA to yield a diagnostic 436 nt cleavage product (Figure 6D). Significantly less target cleavage was observed for the siRNA containing a 5′ blocked anti-sense strand (anti-sense CH_3O-dT; sense, HO-rC). Since it is the sense strand that guides anti-sense target cleavage, these data imply that recognition of the 5′ phosphates of both siRNA strands occurs during the RNAi pathway. In support of this idea, an siRNA in which the anti-sense strand is 5′ dT was also less efficient in anti-sense target cleavage than a siRNA duplex with a rU at this position (data not shown). For the siRNA sequence examined here, the 5′ phosphate of the nonguiding strand contributes to cleavage efficiency, whereas the 5′ phosphate of the target-complementary, guide strand is required for cleavage. The difference in effect of a 5′ phosphate on the guide versus the nonguiding strand suggests that two distinct 5′ phosphate-recognition steps occur in the RNAi reaction.

To test the idea that one of these 5′ phosphate recognition steps precedes assembly of a protein complex on the siRNA duplex, we repeated the experiments in Figure 4 using synthetic siRNA duplexes containing either 5′ hydroxyl or 5′ phosphate groups. siRNAs, radiolabeled on the 3′ end of the anti-sense strand, were incubated in a standard in vitro RNAi reaction in the presence or absence of ATP, then fractionated by gel filtration. siRNAs were detected by scintillation counting of the

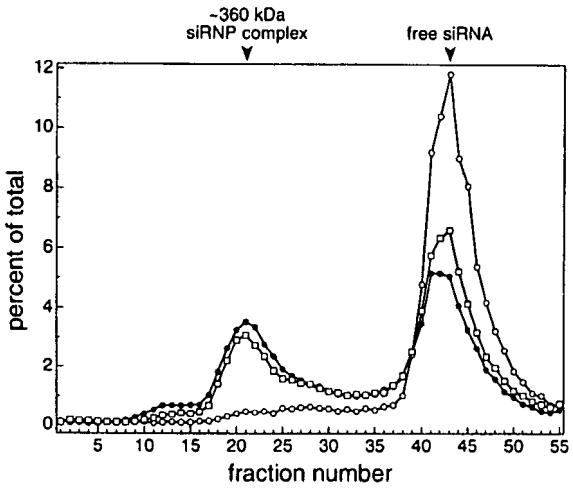

Figure 7. 5′ Phosphates Are Required for siRNP Formation

Complex formation was monitored by gel filtration on Superdex-200. Filled circles, siRNA duplex bearing 5′ hydroxyl groups incubated with *Drosophila* embryo lysate and ATP. Open circles, siRNA duplex bearing 5′ hydroxyl groups incubated in the absence of ATP. Open squares, siRNA duplex bearing 5′ phosphate groups incubated in the absence of ATP.

gel filtration fractions (Figure 7). As shown previously (Figure 4D), synthetic siRNA bearing 5′ hydroxyl groups are incorporated into a ~360 kDa complex upon incubation with *Drosophila* embryo lysate in the presence of ATP (Figure 7, filled circles). However, in the absence of ATP, no such complex is observed, and virtually all the siRNA remains unbound by protein (Figure 7, open circles). These results contrast with those of Figure 4C, in which purified native siRNAs, generated by cleavage of long dsRNA, readily assembled with proteins in the absence of ATP to yield a ~360 kDa complex. Unlike the synthetic siRNA duplexes used in Figure 7, native siRNAs contain 5′ phosphate groups. Thus, one explanation for our results is that 5′ phosphates are required for incorporation of siRNA duplexes into the ~360 kDa complex. In support of this idea, 5′-phosphorylated, synthetic siRNA duplexes form the ~360 kDa complex upon incubation in lysate in the absence of ATP (Figure 7, open squares). These results suggest that the ~360 kDa siRNA-protein complex, although inactive for target cleavage, is a bona fide intermediate in the assembly of the RISC*, the active siRNA complex. Furthermore, they argue that 5′ phosphate recognition occurs early in the RNAi pathway, since we detected no stable siRNA-protein complexes for siRNAs lacking a 5′ phosphate. Interestingly, normal levels of ~360 kDa complex were formed with an siRNA containing a 5′ blocked (CH_3O, dT) anti-sense strand paired with a 5′ hydroxyl sense strand (HO, rC), suggesting that a 5′ phosphate on one of the two siRNA strands is sufficient for siRNP formation (data not shown) and consistent with our finding that a 5′ PO_4, dT anti-sense siRNA strand paired with a 5′ HO, dC sense strand mediates efficient sense target cleavage (see above).

A Model for the RNAi Pathway

Our data suggest that ATP plays at least three distinct roles in the RNAi pathway. In Figure 8, we propose a

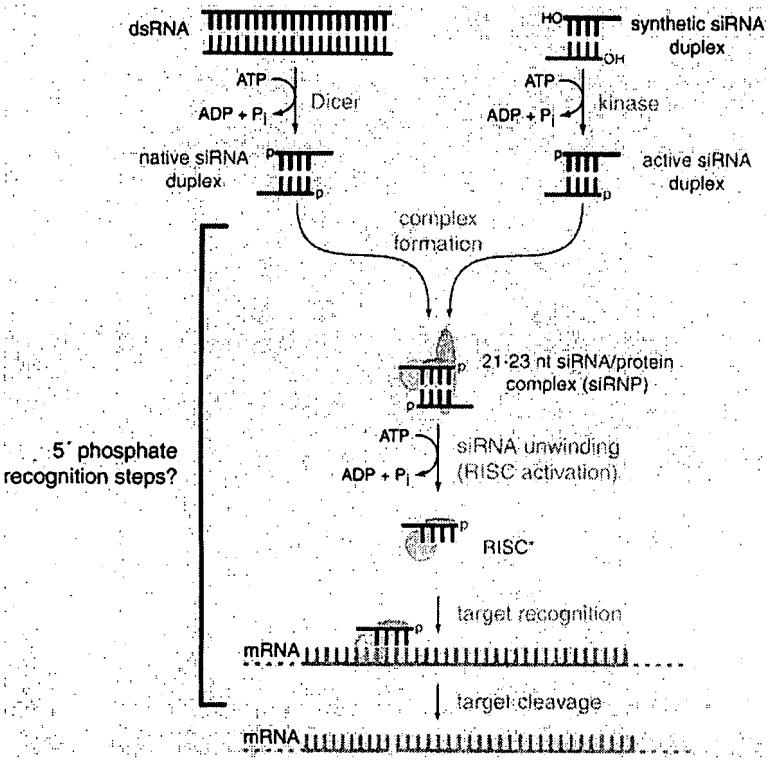

Figure 8. A Model for the RNAi Pathway

We do not yet know if only one or both siRNA strands are present in the same RISC* complex.

model for RNAi that incorporates these three ATP-dependent steps. First, as previously reported, ATP is essential for the cleavage of long dsRNA into native siRNAs. These siRNAs are fully competent to direct RNAi, since when purified, they reenter the RNAi pathway so long as their double-stranded character is maintained. siRNAs are then proposed to bind specific proteins that commit them to the RNAi pathway. In *Drosophila* embryo lysate, the majority of siRNAs are incorporated into a ~360 kDa complex. Although this complex is not competent to direct target cleavage, it seems likely that it is an intermediate in the RNAi pathway, since it is not formed with siRNAs that do not mediate RNAi because they lack 5′ phosphates. Conversion of the inactive ~360 kDa complex into an active complex, RISC*, is proposed to occur in a second ATP-dependent step: the unwinding of the siRNA duplex by an RNA helicase. We do not yet know if the two unwound, single strands are retained in the same complex, or if a single RISC* contains only one of the two strands of the original siRNA duplex. siRNA unwinding is likely to be a stable rather than a transient change in siRNA conformation, because siRNA duplexes preincubated with lysate and ATP are competent to recognize and cleave a corresponding target RNA after extensive dialysis to remove ATP and other cofactors.

5′ phosphorylation of siRNAs corresponds to a third ATP-dependent step in the pathway. Our experiments with synthetic siRNAs reveal the requirement for a 5′ phosphate on the siRNA strand complementary to the target RNA, and a partial requirement for 5′ phosphorylation of the siRNA strand sharing sequence with the target RNA. Tuschl and coworkers have proposed that the site of cleavage of the target RNA is measured from the 5′ end of the complementary siRNA strand. They find that additional nucleotides at the 3′ end of the siRNA do not alter the site of cleavage of the target RNA, but additional nucleotides at the 5′ end move the target cleavage site correspondingly (Elbashir et al., 2001c). The 5′ phosphate of the siRNA may therefore serve as a molecular reference point from which the cleavage site is measured. 5′ phosphorylation of synthetic siRNA duplexes in the *Drosophila* embryo lysate is catalyzed by a kinase that can discriminate between 5′ ribo and 5′ deoxy siRNAs. Might the kinase that phosphorylates synthetic 5′ hydroxyl-containing siRNAs also act on the native siRNAs generated by processing of long dsRNA? While we have not yet devised methods to follow a single phosphate from long dsRNA into an individual siRNA sequence, we note that in the lysate, the half-life of a 5′ ^{32}P on a synthetic siRNA is short, yet virtually all of these siRNAs are 5′ phosphorylated throughout the reaction. The 5′ phosphate of siRNAs generated by the cleavage of long dsRNA may also be exchanging rapidly, with an siRNA-specific kinase serving to regenerate functional siRNA duplexes. We propose that this kinase acts in vivo to maintain the 5′ phosphates of siRNAs, thereby allowing them to participate in multiple rounds of target cleavage.

Why should the RNAi machinery examine the phosphorylation status of an siRNA? Three features—a 21–23 nt length, a double-stranded structure with 2 nt 3′ overhangs, and 5′ phosphates—distinguish siRNAs in flies from other small RNAs, and therefore allow the cell to discriminate between authentic siRNAs and imposters. In this view, the 5′ phosphate is one feature that licenses an siRNA for RNAi. An siRNA-specific kinase would maintain 5′ phosphates on bona fide siRNAs that have

entered the RNAi pathway but have subsequently lost their 5′ phosphate, but would not add 5′ phosphates to other small RNAs, ensuring that only authentic siRNAs target mRNAs for cleavage.

Experimental Procedures

General Methods

Drosophila embryo lysate preparation, in vitro RNAi reactions, dsRNA processing reactions, 501 bp *Rr*-luc and 505 bp *Pp*-luc dsRNAs, full-length *Rr*-luc and *Pp*-luc target mRNAs, and cap-radiolabeling of target RNAs with guanylyl transferase were as described (Tuschl et al., 1999; Zamore et al., 2000). In the experiment shown in Figure 4D, the control RNA was a 441 nt fragment of the *Drosophila pumilio* cDNA transcribed with T7 RNA polymerase from a PCR template prepared with the following primers: 5′ primer, GCG TAA TAC GAC TCA CTA TAG GCG CCC ACA ATT GCC ATA TC; 3′ primer, AAG GTT GAG CCT ACG GCT C. The 510 bp *Rr*-luc target RNA was transcribed with T7 RNA polymerase from a PCR template prepared with the following primers: 5′ primer, GCG TAA TAC GAC TCA CTA TAG GAA AAA CAT GCA GAA AAT GC; 3′ primer, GAA GAA TGG TTC AAG ATA TGC TG. The 510 bp *Pp*-luc sense target RNA was transcribed from a PCR template prepared with the following primers: 5′ primers, GCG TAA TAC GAC TCA CTA TAG GAG ATA CGC CCT GGT TCC TG; 3′ primer, GAA GAG AGG AGT TCA TGA TCA GTG. For transcription templates for the 510 nt *Pp*-luc anti-sense target RNA, the PCR primers were GCG TAA TAC GAC TCA CTA TAG GAG AGG AGT TCA TGA TCA GTG (5′ primer) and GAA GAG ATA CGC CCT GGT TCC TG (3′ primer). Gels were dried and exposed to image plates (Fuji or Kodak), which were scanned using a Bio-Rad Personal FX imager and analyzed with QuantityOne 4.0 (Bio-Rad). Images for figures were prepared with QuantityOne 4.0 and PhotoShop 5.5 (Adobe). Graphs were prepared and rates determined using Microsoft Excel and IgorPro 3.1 (Wavemetrics).

Synthetic siRNAs

The siRNA duplexes in Figures 4B, 5D, 6, and 7 were prepared from synthetic 21 nt RNAs (Dharmacon Research). Sense siRNA sequences were 5′-HO-CGU ACG CGG AAU ACU UCG AUU-3′ (HO-rC) and 5′-HO-dCGU ACG CGG AAU ACU UCG AUU-3′ (HO-dC). Anti-sense siRNAs used were 5′-HO-UCG AAG UAU UCC GCG UAC GUG-3′ (HO-rU); 5′-CH_3O-dTCG AAG UAU UCC GCG UAC GUG-3′ (5′ blocked; CH_3O-dT); and 5′-HO-dTCG AAG UAU UCC GCG UAC GUG-3′ (HO-dT). siRNAs were deprotected according to the manufacturer's instructions, dried in vacuo, resuspended in 400 μl water, dried in vacuo again, resuspended in water, and annealed to form duplex siRNAs as described (Elbashir et al., 2001a). siRNA duplexes were used at 50 nM final concentration. siRNA strands were phosphorylated with PNK (New England Biolabs) and 1 mM ATP according to the manufacturer's directions. siRNAs were 3′ end-labeled with α-^{32}P-cordycepin 5′ triphosphate (5000 Ci/mmol; New England Nuclear) and Poly(A) polymerase (Life Technologies) according to the manufacturer's instructions. Radiolabeled siRNA strands were purified from 15% denaturing acrylamide gels. 5′ phosphorylation status was monitored by electrophoresis of siRNAs on a 15% sequencing gel.

Gel Filtration of Native siRNAs and siRNP Complexes

Preparative scale dsRNA processing reactions (1 ml) were deproteinized at room temperature by the addition of 1 ml 2× PK buffer (200 mM Tris-HCl [pH 7.4], 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate) and Proteinase K (E.M. Merck; 20 μg/μl dissolved in water) to a final concentration of 1 μg/μl. After incubation at room temperature for 1 hr, the reaction was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the RNA recovered by precipitation with 3 volumes absolute ethanol. The precipitate was redissolved in 250 μl lysis buffer (30 mM HEPES-KOH [pH 7.5], 100 mM potassium acetate, 2 mM magnesium acetate) and chromatographed at room temperature on a Superdex-200 HR 10/30 column (Pharmacia) at 0.75 ml/min in lysis buffer using a BioCad Sprint (PerSeptive Biosystems). After 7.5 ml had passed through the column, sixty 200 μl fractions were collected using a cooled stage to maintain the fractions at 4°C. To compensate for dilution of the original reaction on the gel filtration column, each fraction was concentrated by precipitation with ethanol and redissolved in 30 μl lysis buffer.

For siRNP analysis, a 200 μl RNAi reaction was assembled using the purified native siRNAs (fractions 44 and 45) or synthetic siRNA duplexes, incubated for 1 hr at 25°C, and fractionated on Superdex-200 HR 10/30 at 0.75 ml/min in lysis buffer containing 1 mM DTT, 0.1 mM EDTA, and 10% (v/v) glycerol. Fractions were collected as described above. To analyze RNAi activity, 6 μl of each fraction was analyzed in a 10 μl standard RNAi reaction.

ATP Depletion

ATP depletion by hexokinase treatment was as described (Zamore et al., 2000), except that 20 mM glucose was used. In Figure 4B, NEM was freshly prepared from powder as a 1 M stock in absolute ethanol, added to an RNAi reaction at 4°C to a final concentration of 10 mM, incubated 10 min, and quenched with 10 mM DTT added from a 1 M stock dissolved in water. In Figure 4D, an RNAi reaction was adjusted to 10% saturation by addition of a 100% saturated solution of ammonium sulfate dissolved in lysis buffer containing 0.1 mM EDTA. After 30 min at 4°C, the reaction was centrifuged at 16,060× g for 20 min at 4°C, and the supernatant adjusted to 45% saturation. After an additional 30 min at 4°C, the 10%–45% ammonium sulfate precipitate was collected by centrifugation. The precipitate was redissolved in lysis buffer containing 2 mM DTT and dialyzed in a microdialysis chamber (Pierce; 10,000 MW cutoff) for 16 hr against two changes of a >5,000-fold excess of lysis buffer containing 2 mM DTT and 20% w/v glycerol. ATP concentration was measured with a Bioluminescent ATP Assay Kit (Sigma) according to the manufacturer's directions. Luminescence was measured in a Mediators PhL luminometer.

siRNA Unwinding Assay

RNAi reactions (10 μl) were quenched with 90 μl of stop mix containing 1.11× PK buffer, 1.11 ug/ul Proteinase K, 20 μg glycogen (Roche), and a 25-fold molar excess (12.5 pmol) of either unlabeled 21 nt RNA identical to the radiolabeled siRNA strand or a 510 nt *Pp*-luc RNA which contains the sequence of the first 19 nt of the radiolabeled siRNA strand, incubated at 25°C for 15 min, and immediately precipitated with 3 volumes absolute ethanol, chilled for at least 1 hr at −20°C, collected by centrifugation, redissolved in 10 μl 3% w/v Ficoll-400, 0.04% w/v Bromophenol Blue, and 2 mM Tris-HCl (pH 7.4), and immediately analyzed by electrophoresis at 10 W at 4°C through a 15% native polyacrylamide gel (19:1, acrylamide:bis-acrylamide) cast in 1× and run in 0.5× Tris-Borate-EDTA buffer.

Acknowledgments

The authors acknowledge Craig Mello and Tom Tuschl for sharing data prior to publication and for numerous helpful conversations, Dave Bartel, Phil Sharp, Reid Gilmore, and Kendall Knight for continuing advice and encouragement, Stephen Scaringe for advice on siRNA design and synthesis, and members of the Zamore laboratory for comments on the manuscript and many helpful discussions. Guanylyl transferase was the kind gift of Jeff Wilusz. P.D.Z. is a Pew Scholar in the Biomedical Sciences. This work was supported by a Cutler Award from the Worcester Foundation for Biomedical Research and by a grant from the National Institutes of Health (GM62862-01) to P.D.Z.

Received August 21, 2001; revised October 3, 2001.

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